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<b>13. SUPPLEMENTARY NOTES</b>				
<b>14. ABSTRACT</b> The hypothesis is that megakaryocytes (MKs) contribute to the growth of metastatic breast cancer in the bone either by preparing a niche and/or by responding to the cytokines of the marrow resulting from the interaction of the cancer cells with cells of the marrow. We found that MK increased in mouse femurs bearing MDA-MB-231 human cancer. We are comparing MKs in femurs in mice with subcutaneous (non-metastasizing) vs bone metastasizing (intracardiac injection) tumors over time. The treatments are complete; femurs are embedded in paraffin and are being sectioned. We established immunohistochemical procedures for the von Willibrand factor and will use this approach to determine MK numbers. In the meantime thymopoietin -/- mouse embryos were regenerated and mice are being backcrossed to Balb/c so that metastasis can be determined in MK deficient mice using a syngeneic system. Mouse mammary tumor cells (4T1.2) will be injected into the mammary glands of WT and TPO-/- mice, and femurs collected over time. Bone cytokines also will be assessed.				
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## INTRODUCTION

Metastasis to bone is a common sequela of breast cancer. Why breast and certain other solid tumors prefer to metastasize to bone versus other organs is not clear. While examining sections of femurs of mice with metastatic human breast cancer cells, we observed that megakaryocytes had significantly increased in number in the marrow of those mice with cancer compared with non-tumor bearing mice[1]. We could find no reports of a direct role for megakaryocytes in metastatic cancer but many indirect connections. For example, megakaryocytes are the source of platelets which release growth and angiogenic factors, and contribute to basement membrane proteolysis [2]. Megakaryocytes differentiate in the endosteal niche, the same place where cancer cells home. In addition megakaryocytes produce many characteristic osteoblastic molecules such as RANKL and OPG that can regulate osteoclast activity. This information taken together led to the hypothesis that megakaryocytes contribute to growth of breast cancer cells in the bone either by preparing a niche and/or by responding to the cytokine microenvironment of the marrow that results from the cancer cells and the osteoblasts/stromal cells. Mouse models, xenograft and syngeneic, will be used to compare megakaryocytes in femurs under conditions of metastasis or non-metastasis. Thrombopoietin (TPO<sup>-/-</sup>) knockout mice will be used to test metastasis in mice with a megakaryocyte deficiency. Megakaryocyte numbers (detected by immunohistochemistry for von Willebrand expression) and secreted bone cytokines will be measured.

## BODY

**Task 1.** Begin the process of creating TPO<sup>-/-</sup>-mice on a Balb/c background.

We had received permission from Genentech to obtain frozen embryos of TPO<sup>-/-</sup> mice. Genentech no longer maintains the line of mice but had stored frozen embryos at Jackson Laboratories. We originally proposed to have the embryos shipped here and the work would be done at Penn State. However, since Jackson Laboratory already had the frozen embryos, it made technical and economical sense to engage Jackson Laboratories to regenerate the frozen embryos and to mate the mice to transfer the TPO<sup>-/-</sup> from the C57Blk6 background onto the Balb/c background. Jackson reported the successful regeneration of the frozen embryos. They have begun the backcrosses. I have copied their most recent email (4/17/2011).

Dear Dr. Mastro,

I have attached the colony update for Speed Congenic project 12994. We have set up the first generation of backcrossing heterozygous TPO females to BALB/cJ males. We are holding 3 TPO heterozygous males until these breeders have pups.

Should you have any questions, please do not hesitate to contact me or Rob Brankamp.

Kind regards,

Phyllis

Phyllis Magnani

JAX<sup>®</sup> Services Project Manager

This task is on schedule.

**Task 2.** Megakaryocytes and bone metastases in a xenograft model, a comparison of megakaryocytes in femurs of mice with bone metastatic cancer compared with cancer in the mammary gland.

1. Optimization of megakaryocyte detection.

Immunohistochemical procedures were optimized for von Willebrand factor. We determined that this procedure gave enhanced detection of megakaryocytes compared with H&E staining (Figure 1).

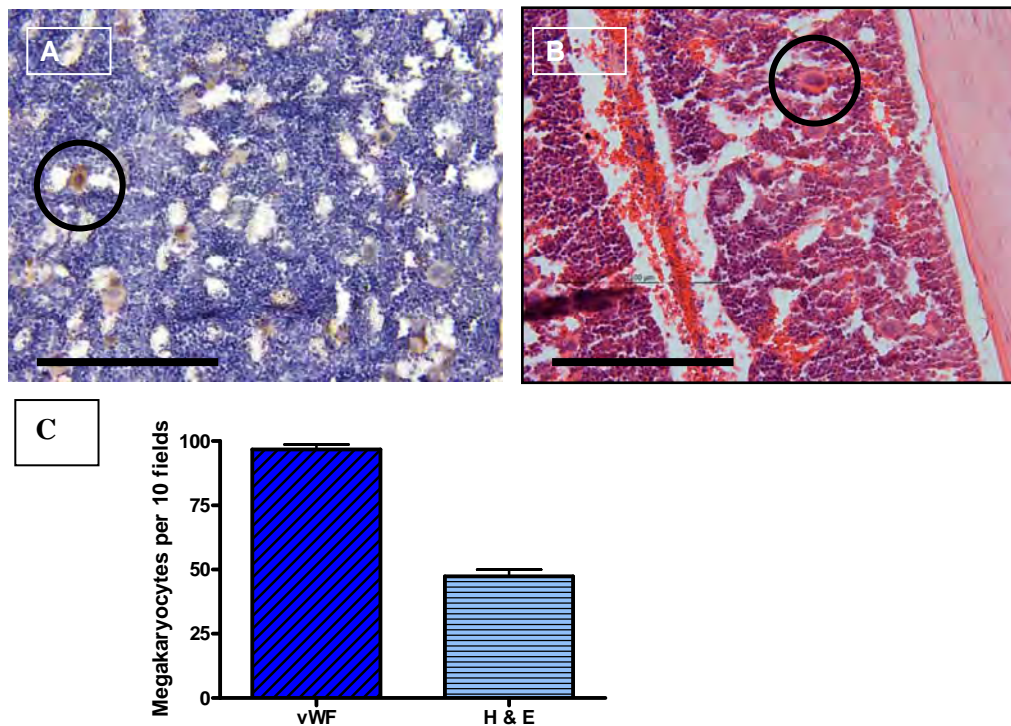


Figure 1. Comparison of megakaryocytes in the bone marrow of femurs detected by immunohistochemistry and von Willebrand factor or hematoxylin and eosin (H&E) staining. Femurs from mice inoculated in the left ventricle of the heart with MDA-MB-231 cells were removed at sacrifice three weeks following inoculation. The bones were fixed with 4% paraformaldehyde, decalcified with EDTA, and embedded in paraffin. They were longitudinally sectioned (10 microns), and placed on polylysine coated slides. **A.** For immunohistochemistry, the sections were dewaxed and antigen retrieval was carried out with a 15 min treatment at 37° with 0.5% trypsin in a humidified chamber. To reduce endogenous peroxidase activity, the sections were incubated with 3% H<sub>2</sub>O<sub>2</sub> for 45 min. The sections were incubated with 10% donkey serum in PBS for 2 hr, incubated overnight with a primary rabbit antibody (10 µg/ml, ABCAM) to von Willibrand factor, washed and incubated with a secondary antibody, biotinylated donkey anti-rabbit (1:1000 in 10% donkey serum) for 2 hr. Antigens were visualized with avidin-conjugated horseradish peroxidase and DAB substrate (Vector Labs). **B.** Sections were processed and stained with H&E with a Shandon Gemini Varistainer. **C.** Enumeration of megakaryocytes stained with H&E or with IHC.

We tested the immunohistochemical protocol on bone sections from tibias of mice that had been inoculated in the heart for another experiment. We found a significant increase in megakaryocytes in bones of mice inoculated with MDA-MB-231 cells or the metastasis suppressed MDA-MB-231BRMS1 variant (Figure 2) two weeks following inoculation. At three weeks that increase was only apparent with the MDA-MB-231 cells.

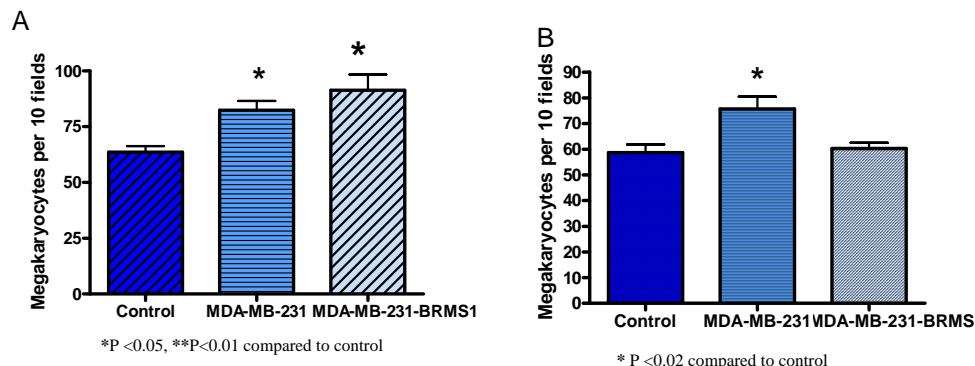


Figure 2 . Megakaryocytes in tibia of mice inoculated in the left ventricle of the heart with MDA-MB-231 or the metastasis suppressed MDA-MB-231BRMS1 cells. Sections of tibias were stained for the presence of von Willebrand's factor and megakaryocytes were counted.

We also attempted to quantify megakaryocytes from both spleen and bone marrow using flow cytometry. The size of the megakaryocytes as well as their rarity compared to the other cells, made this method impractical.

## 2. Pilot experiment to determine the use of luciferase expressing MDA-MB-231 cells lines.

We had proposed to use GFP expressing cancer cells to detect the presence of cancer colonies in bones. However, we determined that the ability to detect metastases in the entire mouse would be enhanced by using luciferase-expressing breast cancer cell lines. We obtained from the laboratory of Dr. Joan Masague bone derivatives of MDA-MB-231 cells, 1833TR and SCP2 [3]. Both express luciferase. We carried out a pilot experiment to determine how well each cell line would grow in the mammary gland and in the bone marrow. The cells were injected at  $10^5$  cells in 100  $\mu$ l PBS for intracardiac inoculation or  $10^6$  cells in 50  $\mu$ l PBS for mammary gland injection into female athymic mice about 6 weeks of age, 6 mice per group. The intracardiac inoculated mice were followed by IVIS live animal imaging at days 3, 10, 16, 24, 31 and 37 days. The mammary gland injected animals were imaged once a week for 5 weeks. The SCP2 grew in the bone but not in the mammary gland. However 1833TR grew both in the mammary glands and in the bones. Therefore the 1833TR, MDA-MB-231 cells were chosen for the experiments described in task 2.

### Task 2a. Quantification of megakaryocytes in femurs of mice inoculated with MDA-MB-231 (1833TR, luciferase) cells in the left ventricle of the heart.

Based on the pilot data we chose the 1833TR luciferase MDA-MB-231 cells. Six mice per group were inoculated in the left ventricle of the heart with the cancer cells ( $10^5$  cells in 100  $\mu$ l PBS) or with 100  $\mu$ l PBS for harvest at times 1, 4, 10, 20 and 30 days following inoculation. The mice were imaged with IVIS immediately after inoculation and again prior to sacrifice. Serum and blood samples were collected at the time of sacrifice. An automated Hemavet instrument was used to perform complete

blood counts. Variations in platelet counts were not statistically significant over time or between groups (Figure 3). Serum is frozen awaiting assay of SDF-1 and TPO. The bones have been fixed and are paraffin embedded. Currently, sectioning of the bones is in progress. A representative IVIS image of one mouse at each time is presented (Figure 4). Inoculations were deemed successful based on luciferase distribution post injection. (Figure 4 Aa, Ba and Ca.) Of the 30 mice inoculated with 1833TR-luc cells, none of them showed visible luciferase activity at 1 or 4 days after inoculation into the heart (Figure 4 A). However, by day 10, 4/6 showed metastasis and 2 of these appeared to be associated with bone. All of the mice at days 20 and 30 following inoculation showed metastasis and 8 out of 16 were obviously associated with bone (Figure 4B, C).

A

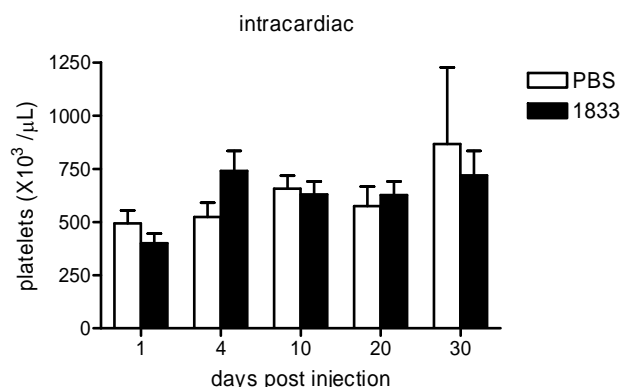
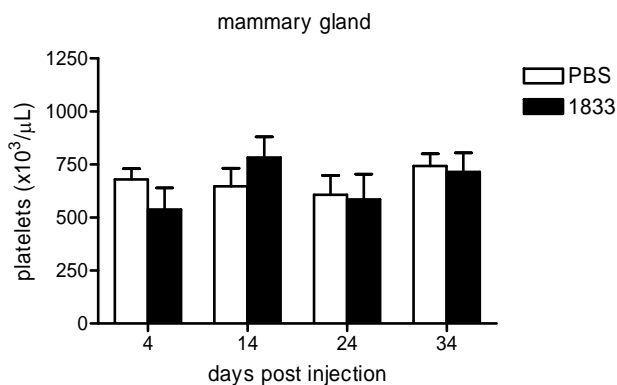


Figure 3. Platelet counts from mice following MDA-MB-231 or PBS inoculation into A. the left ventricle of the heart or B. the mammary gland. Blood was collected at the time of sacrifice as indicated. Shown are the averages  $\pm$  the SD for 6 mice.

B



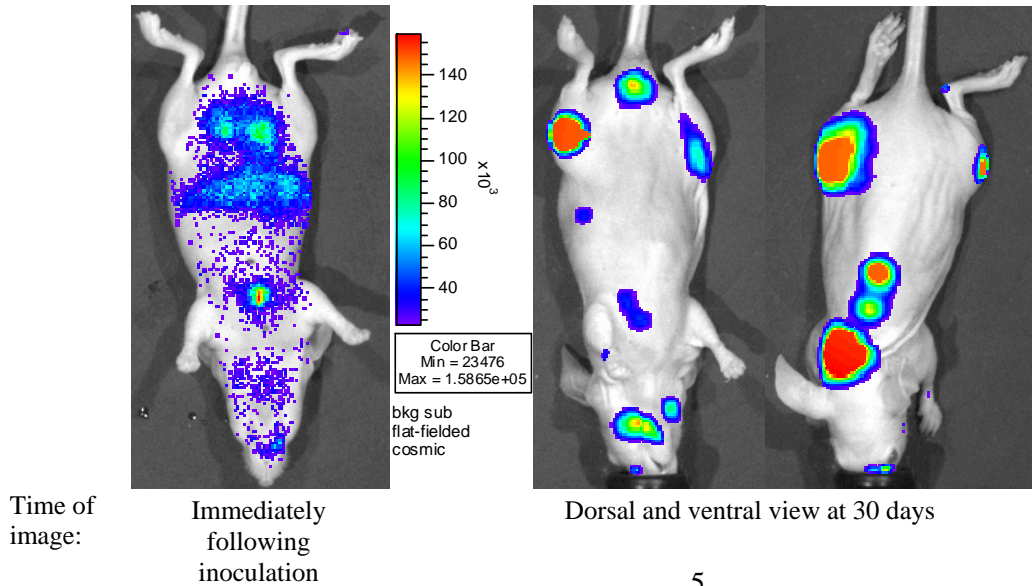
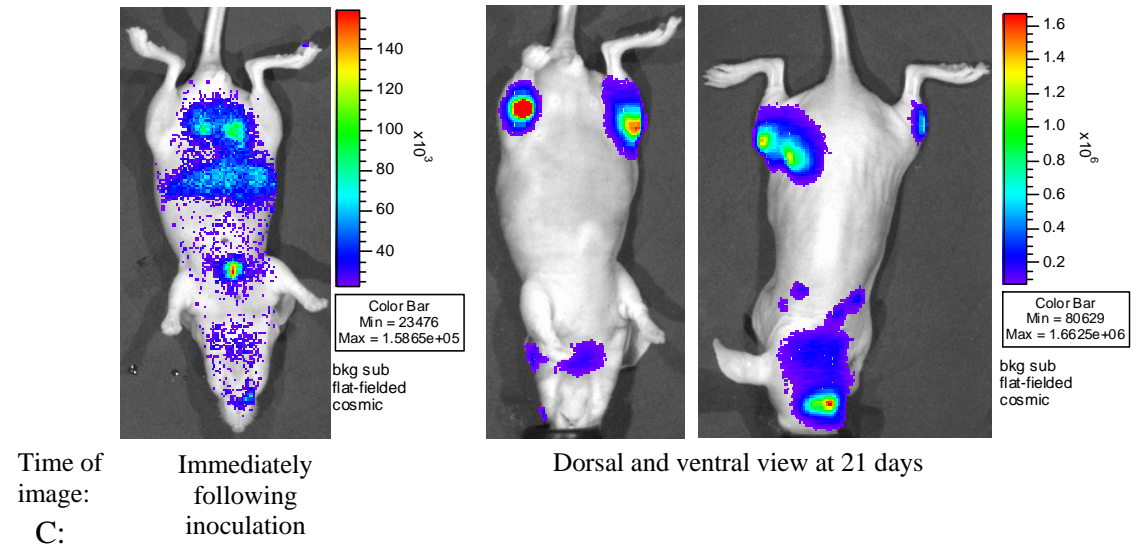
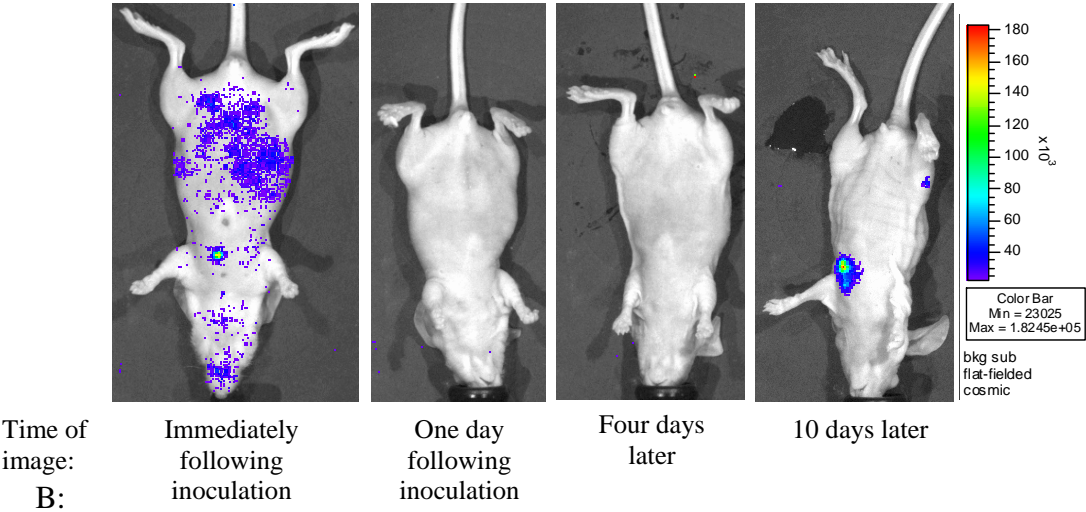
In order to measure SDF-1 and TPO in the same samples, we are developing a multiplex assay with support from Mesoscale Discovery Corporation. The assay requires only 50  $\mu$ L of serum from each mouse. Screening for antibody pairs is underway.

**Task 3a: Quantification of megakaryocytes in femurs of mice inoculated with MDA-MB-231 (1833TR, luciferase) cells in the mammary gland.**

Six mice per group were injected in the fourth mammary gland with  $10^6$  cells in 50  $\mu$ L PBS or with PBS only. They were sacrificed at days 4, 14, 24 and 34. IVIS imaging was carried out prior to sacrifice. Whole blood and serum samples were collected for CBC (including platelet count), SDF-1 and TPO analysis. Femurs were collected and are being processed as in task 2a. Based on IVIS

imaging the 1833 cells grew in the mammary glands of 21 of the 24 mice injected (Figure 4D). The mammary tumors grew larger over time but metastases were not apparent.

A:





D:

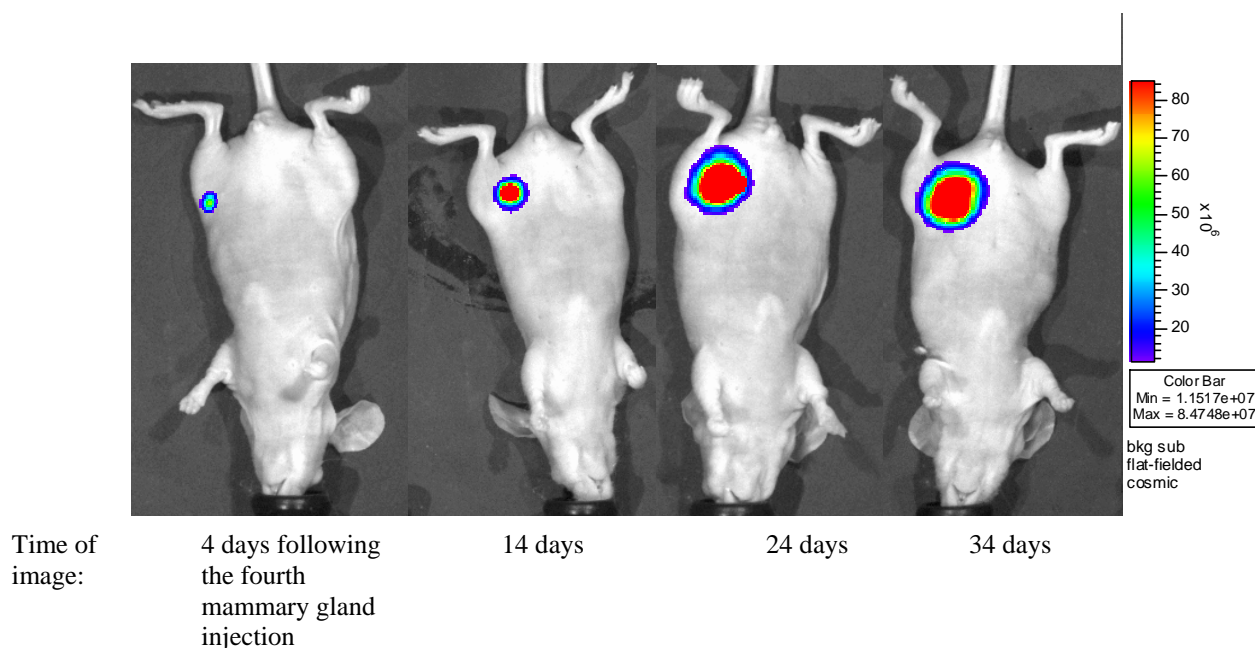


Figure 4. IVIS imaging of mice inoculated with MDA-MB-231-luc (1833TR) cells in the left ventricle of the heart or in the mammary gland. Athymic mice were inoculated with cancer cells,  $10^5$  for intracardiac or  $10^6$  for mammary glands, or with PBS. Immediately following inoculation and at the times indicated following inoculation, the mice were imaged with IVIS to detect the luciferase activity. **A-C.** Intracardiac; **D.** Mammary gland.

### Supportive in vitro experiments using a human megakaryocyte cells line, MEG-01.

In order to investigate the effects that specific cells of the metastatic bone microenvironment may exert on megakaryocyte proliferation and differentiation, we designed several *in vitro* experiments using the human megakaryoblastic cell line, MEG-01 [4]. These cells can differentiate along the megakaryocyte lineage and produce platelet-like particles in the presence of phorbol myristate acetate (PMA).

Conditioned media (RPMI-based) were obtained from four cell types representing those that are present in the metastatic bone microenvironment: 1) hFOB (human fetal osteoblasts) [5]; BMEC (human bone marrow endothelial cells) [6]; 3) MDA-MB-231 (metastatic breast cancer); 4) MEG-01 (human megakaryoblasts). These media were then added at 50% concentration to MEG-01 cultures. Three parameters were examined: proliferation, ploidy (number of nuclei) and mRNA expression of megakaryocyte differentiation markers, CD61, CD62p and thromboxase (TXS) [7].

For proliferation experiments, MEG-01 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 U/ml penicillin, 100 µg/ml streptomycin. Cells were counted and plated at a concentration of 50,000 cells/well, 96-well plates. After allowing cells to attach overnight, each of the conditioned media types supplemented with 10% fetal bovine serum was added. After 1, 2 and 3 days MTT (cell viability) assays were performed on select wells. Absorbance was measured at 550nm. All samples were run in duplicate.

The conditioned media from all the cell types including MEG-01 suppressed the proliferation of the MEG-01 cells when compared to cells grown in the vehicle medium (Figure 5). By day 3 of culture, the cells treated with the breast cancer cell conditioned medium showed the greatest effect.

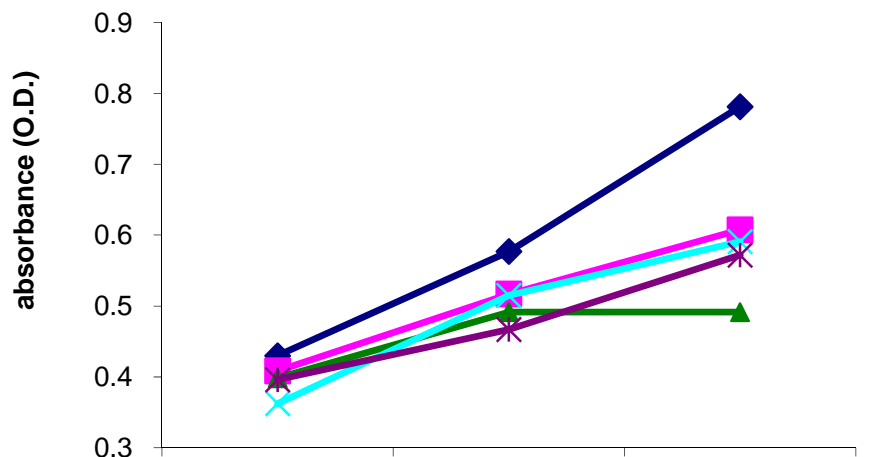


Figure 5 Proliferation of MEG-01 megakaryocytes cultured in conditioned media from cells of the metastatic bone microenvironment. MEG-01 cells were grown in RPMI 1640, 10% fetal bovine serum and 100 U/ml penicillin, 100 µg/ml streptomycin. Cells were plated initially at a concentration of 50,000 cells/each well of a 96-well plate. After overnight incubation, conditioned media (hFOB-osteoblasts; MDA-MB-231-metastatic breast cancer cells); BMEC-bone marrow endothelial cells) supplemented with 10% fetal bovine serum were added. Normal growth media (vehicle medium VM) was used as a control. At 1, 2 and 3 days after addition of the conditioned media, MTT viability assays were performed. Samples were run in duplicate. The highest level of MEG-01 proliferation was seen with VM (blue square) while the lowest level of proliferation was seen with MDA-MB-231 conditioned media (green triangle).

To determine formation of multinuclei, MEG-01 cells were plated in 50% growth media and 50% conditioned medium from immature hFOB, mature hFOB, MDA-MB-231, BMEC or MEG-01 cells in 6 well plates. The addition of 10nM PMA to growth media served as a positive control. Cultures were incubated at 37°C for 3 days. Cells were harvested, microcentrifuged onto glass microscope slides using a Shandon Cytospin, and stained using a modified Wright-Giemsa stain. Nuclei per cell were counted for 25 cells per slide for biological duplicates.

The conditioned media from all cell types examined appeared to increase the number of multinucleated megakaryocytes when compared to vehicle medium (Figure 6). However, the conditioned media from mature osteoblasts had the greatest impact on megakaryocyte maturation; 32% 4n, 4% 6n and 4% 8n when compared with 20% 4n, 2% 6n and 0% 8n for controls. Conditioned media from MDA-MB-231 cancer cells and BMEC cells showed the least effect (24% 4n, 6% 6n and 22% 4n and 4% 6n, respectively). These data support the idea that osteoblast and megakaryocyte development are tied closely together. While breast cancer cell media directly exerted very little effect on the megakaryocyte development, perhaps there is a cascade in which cancer cells disrupt osteoblast function which in turn interrupts signaling between osteoblasts and megakaryocytes.

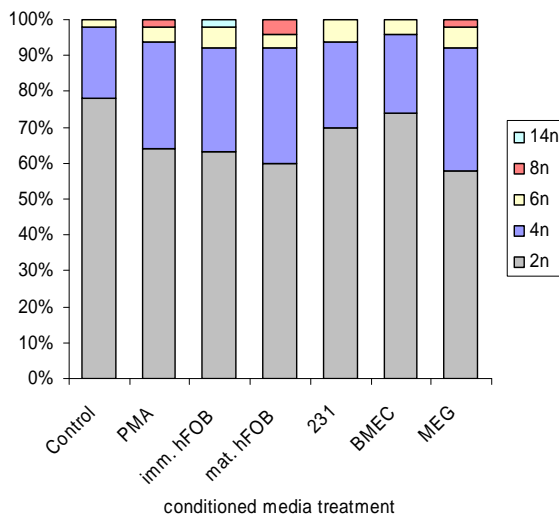


Figure 6. Number of nuclei per megakaryocyte in MEG-01 cells treated with conditioned media. MEG-01 cells were plated in 50% growth media and 50% of conditioned medium from immature hFOB, mature hFOB, MDA-MB-231, BMEC or MEG-01 (control = growth media) in 6 well plates. The addition of 10nM PMA to growth media was used as a positive control. After 3 days, cells were harvested, adhered to glass microscope slides via cytopsin, and stained using a modified Wright-Giemsa stain. Nuclei per cell were counted for 25 cells per slide for biological duplicates. All conditioned media treatments resulted in increases in ploidy over the control treatment. Conditioned media from osteoblasts (hFOB) yielded the highest degree of ploidy; mature hFOB superceded the PMA positive control treatment (PMA 30% 4n, 4% 6n, 2% 8n versus mature hFOB 32% 4n, 4% 6n, 4% 8n).

Three molecules associated with platelet production are temporally express in developing megakaryocytes. Thromboxane synthase (TXS) and glycoprotein IIIa (CD61) are expressed early in the maturation process while the adhesion molecule p-selectin (CD62p) is expressed later. We utilized real time PCR to look for differences in expression of these three molecules in MEG-01 cells that had been treated with conditioned media. Briefly, Meg-01 cells were harvested and resuspended in medium consisting of 50% growth media and 50% conditioned medium from hFOB, MDA-MB-231, BMEC or MEG-01. PMA treated cells were used as a positive control. Cells were incubated at 37°C for 24 hours at which time cells were harvested, RNA extracted and cDNA synthesized. Gene specific primers were used to amplify sequences using a Sybr green /ROX amplification kit and Applied Biosystem 7300 instrumentation. All results were normalized to GAPDH and compared to MEG-01 conditioned media treatment using the  $2^{-\Delta\Delta C_t}$  method to obtain fold changes.

Expression of all 3 molecules was the greatest in the presence of fresh media (VM) (Figure 7). Despite the fact that conditioned media treatments contained only 50% fresh media, CD61 and TXS expression remained high when treated with either hFOB (osteoblast) or BMEC (endothelial cell) media. The adhesion molecule CD62p, however, was not appreciably up regulated by any of the conditioned media. The presence of breast cancer cell conditioned media appeared to reverse the effect of fresh media on the expression of all three molecules. Because breast cancer are known to cause thrombocytosis, this result seems at first glance contrary to our hypothesis. However, due to the plethora of intercellular signaling that occurs in the bone microenvironment, breast cancer cells may not exert a direct effect on megakaryocytes, but rather operate through other cell types. Further experiments into megakaryocyte development are necessary to include media that has been conditioned through multiple cell types.

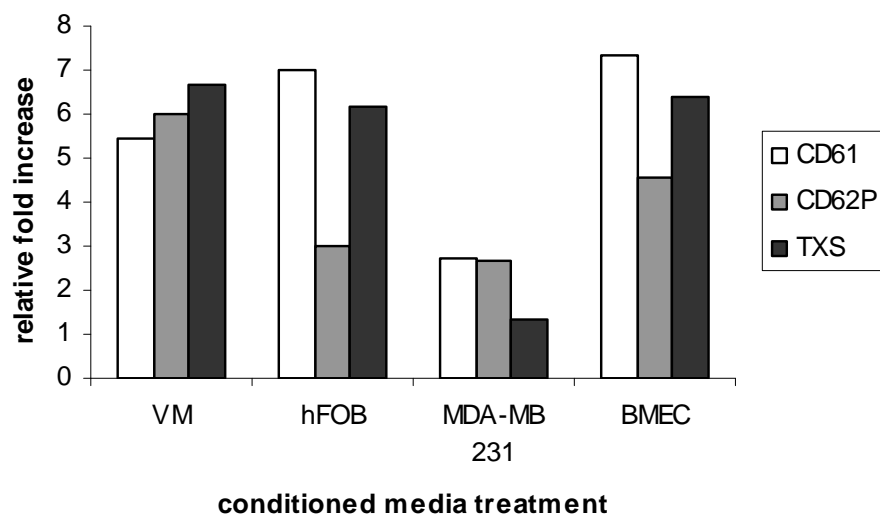


Figure 7. RT-PCR of megakaryocyte maturation genes CD61 (glycoprotein IIIa), CD62p (p-selectin) and TXS (thromboxane synthase) in MEG-01 cells treated with conditioned media. Meg-01 cells were suspended in medium consisting of 50% growth media and 50% conditioned medium from hFOB, MDA-MB-231, BMEC or MEG-01. After 24 hour incubation at 37°C cells were harvested followed by RNA extraction and first strand synthesis. Gene specific primers and real time PCR was performed to determine relative changes in mRNA. Sample results were normalized to GAPDH and compared to MEG-01 conditioned media treatment

#### 4. Task 4. Syngeneic mouse model.

This task has not begun. However, we now have luciferase expressing 4T1.2 and 67NR cells. They will be used instead of lacZ expressing cells. This change will allow us to IVIS image live animals. We can use an antibody to luciferase to detect tumor cells in sections of bone. This obviates the need for task 4a.

- Transfect 4T1.2 and 67NR cells with a plasmid containing the lacZ gene under the control of a CMV promoter and a gene for resistance to puromycin. (month 9).
- Inject cells into the mammary glands of Balb/c mice. Euthanize mice 1,2,3,4 and 5 weeks later. Collect blood and perform platelet counts. Examine for metastases. Remove femurs and treat as in task 1, A.2 except the sections will be incubated with X-gal to detect the tumor cells. (months 10-11).
- Examination of slides and counting of megakaryocytes and cancer cells (month 12-13).
- ELISAs of serum and multiplex cytokine analysis of bone culture supernatants (month 14).

#### 5. Task 5 . Metastasis in TPO knockout model (beginning at month 18).

- Once the TPO knockout congenics have been made they will be treated as in Task 4. (months 18-23).

See task 1. Derivation of the animals has begun.

- 6. Task 6.** Alternative to Task 5. If for some reason it appears that we are unable to breed the congenic TPO<sup>-/-</sup> mice, we will use the wild type Balb/c and treat them with an Anagrelide metabolite to reduce the numbers of megakaryocytes. (months 18-23)
- 7. Task 7.** Data analysis. Manuscript preparation (months 23-24).

## KEY RESEARCH ACCOMPLISHMENTS

- Successful re-derivatization of TPO<sup>-/-</sup> mice and beginning of transfer to a BALB/c background.
- Developed procedure for processing of femurs and immunohistochemistry for von Willebrand's factor.
- Completion of animal inoculations intracardiac and into mammary gland; images taken; femurs in paraffin awaiting immunohistochemistry and counting of megakaryocytes.
- Completed a series of in vitro experiments with a megakaryocyte cell line that osteoblasts stimulate megakaryocyte maturation as indicated by ploidy formation.

## REPORTABLE OUTCOMES

### Manuscripts

No manuscripts have been written yet because we do not have complete data.

### Abstracts

An abstract has been submitted to the Era of Hope Meeting to be held in August of 2011. The Role of Megakaryocytes in Breast Cancer Metastasis to Bone. Mastro, A.M, D.M. Sosnoski and W.A. Jackson. (see appendix).

Stiffen, R M. A.M.Mastro. 2010 The Presence of Megakaryocytes in Cancerous Prostate Cancer Tissue. Science of Global Prostate Cancer Disparities in Black Men. Aug 27-29, Jacksonville, Florida

Miles, J.S, D.M Sosnoski, R.M Stiffen, and Andrea M. Mastro. The Role of Megakaryocytes in Breast Cancer Metastasis to Bone, 2010, Poster Presentation at the Summer Undergraduate Research Program at the Pennsylvania State University.

Miles, J.S, D.M Sosnoski, R.M Stiffen, and Andrea M. Mastro. The Role of Megakaryocytes in Breast Cancer Metastasis to Bone, 2010, Presentation to the REU program, Physics, Penn State University

Kyle, Alvin, R.M. Stiffen, A. M. Mastro, D.M. Sosnoski. 2011. The Role of CD41<sup>+</sup> and von Willebrand Factor + Megakaryocytes in Bone Marrow of Nude, Athymic Mice Injected with Metastatic and Non-metastatic Breast Cancer Cells. FGLSAMP Expo, February 3-6.

Stiffen, R.M. D.M. Sosnoski, A. M. Mastro. (submitted) The Role of CD41+ and von Willebrand Factor + Megakaryocytes in Bone Marrow of Nude, Athymic Mice Injected with Metastatic and Non-metastatic Breast Cancer Cells. Fourth Health Disparities Conference, New Orleans, Louisiana, March 27-029, 2012.

## **Training**

Several undergraduate students and a graduate student have worked on various aspects of this project thus far.

Megan Manno, a Biology major at Penn State, learned how to process and section bone. She also learned how to carry out hematoxylin and eosin staining. She counted megakaryocytes for some of the preliminary data.

She is now completing a post-bac program at the NIH and is applying to medical schools.

Mariella Disturco, a Biochemistry and Molecular Biology Major, learned from Megan. She counted megakaryocytes in sections from bone that we had saved from an unrelated experiment. Mariella also learned how to process and section bone. Mariella will attend medical school in the fall.

Katherine Phillips, a Biochemistry and Molecular Biology Major, carried out all the experiments with MEG-01 cells. She graduated from Penn State in the Spring of 2011. Katie is seeking a position with a Pharmaceutical company.

In the summer of 2010, Dr. Rose Stiffen, a visiting professor from Florida Memorial University (an historically black university, spent 10 weeks in my laboratory. One of her students, Jana Miles, joined the lab as a summer NSF REU student. Together they optimized the immunohistochemistry for von Willebrand's factor. Jana presented her work as a poster during a summer research presentation at Penn State and a talk to the other REU participants. Rose was invited to present her work at the "Science of Global Prostate Cancer Disparities in Black Men" conference, Aug 27-29, 2010, Jacksonville, Florida

Walter Jackson III has joined the laboratory as a Ph.D. student. He has an MS from Alcorn University through the Bridges program with Penn State. He will be carrying out the bulk of the studies associated with the remaining tasks. Walter has inoculated athymic mice in the heart and mammary gland. He has carried out IVIS imaging, sacrificed mice and harvested femurs. He has fixed and processed the bones and is currently preparing sections. He has learned how to carry out the immunohistochemistry.

## **CONCLUSIONS**

Thromboembolism is one of the most common causes of death in cancer patients [8]. Indeed increased platelets are a poor prognostic factor for breast cancer metastasis [9]. Consistent with this information is the observation that we made in a mouse model, that megakaryocytes were increased in the marrow of metastasis bearing mice [1]. We speculated that megakaryocyte either result from bone metastasis or play a role in allowing tumor colonization of bone. We designed animal experiments to test between these two possibilities. The work is in progress. Task 2 and 3 are almost complete. Breeding of the TPO-/- mice is on track.

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## Appendix

### Era of Hope Abstract

#### The Role of Megakaryocytes in Breast Cancer Metastasis to Bone Mastro, A.M, D.M. Sosnoski and W.A. Jackson.

Breast cancer cells frequently metastasize to bone. Despite the clinical ramifications, very little is understood about the fundamental mechanisms responsible for this phenomenon. While examining sections of bone from mice that had been inoculated with metastatic MDA-MB-231 breast cancer cells, we observed a marked increase in the numbers of megakaryocytes (MK) in the bone marrow. We hypothesized that MK contribute to the growth of breast cancer cells in the bone either by preparing a niche for the metastases and/or by responding to the altered microenvironment of the marrow that results from the presence of the cancer. MKs are cells of the hematopoietic lineage that are found in the bone marrow and mature to produce platelets. There is ample evidence that MK and the cytokines and growth factors they produce play a crucial role in bone metabolism and skeletal homeostasis. MKs normally comprise a small percentage of the marrow population, but can increase several fold under stress which correspondingly increases the numbers of circulating platelets. Thrombocytosis is a hallmark of many types of metastatic cancer; thromboembolism is a major cause of death among cancer patients.

The aims of this ongoing study are to determine 1. if the increase in MK precedes the growth of cancer cells in the bone marrow; 2. the role the cancer cell-osteoblast/stromal cell interactions play in the increase in megakaryopoiesis. We will compare MK in the femurs of athymic mice inoculated intracardiacally (metastasis) with those inoculated in the mammary gland (no metastasis). For the second aim we will use a syngeneic model of Balb/c with 4T1.2 cancer cells that metastasize from the mammary gland. We will use luciferase labeled cells to follow growth and metastasis. Serum will be sampled for thymopoietin, SDF-1 and platelets. Finally, Balb/c mice lacking TPO and thus with low MK, will be tested for metastasis. We are in the process of preparing the appropriate congenic strain.

In pilot studies carried out thus far we determined that immunohistochemistry of femur sections with von Willebrands factor allowed us to detect about 50% more MK than H&E staining. The tibias of mice with MDA-MB-231 bone metastases had more MK/field than uninoculated mice. MK increased over time following inoculation of cancer cells from 1 hr through 72 hr. When mice were inoculated in the flank (no metastases), the number of MK in the bone marrow increased proportionally to the size of the tumor.

These studies indicate the importance of MK in the metastatic process.